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ORIGINAL PAPER

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# Purification and characterisation of a lactococcal aminoacylase

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**Abstract** The *amd1*-encoded aminoacylase from *Lactococcus lactis* MG1363 was cloned and overexpressed in *Escherichia coli* and purified. The assumed dimeric enzyme has a subunit molecular mass of about 42 kDa and contains 2.0±0.1 g-atoms of zinc and cobalt, in equimolar amounts, per subunit of Amd1. The enzyme was characterised with respect to substrate specificity, pH, temperature and metal dependence. Amd1 exhibited a broad activity range towards N-acetylated-L-amino acids with a strong preference towards those containing neutral aliphatic and aromatic side chains. It hydrolysed N-acetyl-L-alanine most efficiently, and exhibited temperature and pH optima of 30 °C and 7.0, respectively. The activity of Amd1 towards N-acetyl-L-alanine was enhanced by the divalent cation $Co^{2+}$, while $Cd^{2+}$ inhibited activity. Interestingly, Amd1 was shown to catalyse the hydrolysis of several dipeptides at pH 7.0, although with reduced $V_{max}$ values as compared to hydrolysis of N-acetylated-L-amino acids. This characteristic has also biological significance since Amd1 was able to complement a growth deficiency in a *L. lactis* triple peptidase mutant.

**Keywords** *Lactococcus lactis* · Aminoacylase · Purification · Kinetic analysis

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## Introduction

The large-scale biochemical production of amino acids like alanine and phenylalanine has become a very powerful and economically valuable industrial process (Tewari 1990; Sakanyan et al. 1993). The discovery and biochemical analysis of enzymes that can hydrolyse N-acetylated amino acids into their unmodified amino acid derivatives has for this reason become increasingly important in terms of biotechnological applicability.

The removal of acyl groups from amino acids is an enzymatic activity (Birnbaum et al. 1952; Anders and Dekant 1994) mainly associated with aminoacylases (N-acyl-L-amidohydrolases; EC 3.5.1.14), while this activity is frequently found in carboxypeptidases, dipeptidases and aminopeptidases. Aminoacylases have been isolated from a wide variety of bacteria and archaea, as well as from plants and animals (Mitta et al. 1992, 1993; Sakanyan et al. 1993; Bartel and Fink 1995; Kempf and Bremer 1996; Pittelkow et al. 1998; Wakayama et al. 1998; Javid-Majd and Blanchard 2000; Linder et al. 2000; Ishikawa et al. 2001; Toogood et al. 2002). In mammals they play a role in xenobiotic detoxification and bioactivation, and are essential in the processing of xenobiotic-derived mercapturates (Anders and Dekant 1994; Uttamsingh et al. 2000). In bacteria, although a physiological function has yet to be firmly elucidated, it has been suggested that aminoacylases are involved in metabolising protein-based growth substrates (Story et al. 2001). Thus, it is not surprising that many organisms from which aminoacylases have been isolated are capable of peptidolytic growth. A gene encoding aminoacylase activity, designated *amd1*, was previously cloned from *Lactococcus lactis* MG1363 and sequenced (Curley and van Sinderen 2000). The *amd1* reading frame encodes a 398-amino-acid residue protein with a calculated subunit molecular mass of approximately 42 kDa. This is in good agreement with the size of other known aminoacylases. Comparison of the *amd1* gene product with protein sequences available in the databases revealed significant similarity (27–36%) to a number of other bacterial

aminoacylases. *amd1* appears not to be expressed in *L. lactis* MG1363, as no aminoacylase activity was detected from crude extracts. The latter is probably caused by the presence of an immobilised copy of an IS*982* element immediately upstream of the *amd1* coding region. *amd1* was overexpressed in *L. lactis* and resultant crude extract assays exhibited a broad substrate specificity that was more pronounced with neutral aliphatic and aromatic amino acids (Curley and van Sinderen 2000).

In this report we describe the overexpression and purification of this lactococcal aminoacylase. The temperature and pH dependence of the enzyme was determined, as well as the specificity of metal binding. Extensive kinetic studies allowed a detailed characterisation of its substrate allegiance, which includes dipeptidase activity. Interestingly, Amd1 complemented the growth deficiency of a *L. lactis* triple peptidase mutant.

## Materials and methods

### Bacterial strains, plasmids and growth conditions

Strains and plasmids used in this study are listed in Table 1. *Escherichia coli* cells were grown in liquid LB medium at 37 °C with vigorous agitation, or on LB medium with 1.5% agar (Sambrook et al. 1989), containing 100 µg ampicillin (Ap) $ml^{-1}$ and 25 µg kanamycin (Km) $ml^{-1}$. *L. lactis* cells were grown at 30 °C in M17 glucose medium (Terzaghi and Sandine 1975) or in chemically defined medium (CDM) (Jensen and Hanuner 1993). Where needed, erythromycin (Em) and chloramphenicol (Cm) were added at final concentrations of 3 µg $ml^{-1}$. For specific experiments, outlined below, CDM was supplemented with Leu-Met, N-acetyl-L-Leu, N-acetyl-L-Leu-Arg-Arg-Ala-Ser-Val-Ala or N-acetyl-L-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln at a final concentration of 1 mM (Sigma-Aldrich).

### Construction of pQEamd1

*amd1* was amplified from *L. lactis* MG1363 chromosomal DNA by PCR, using two primers (5′ AAGTCCATGGAACTACTTAATA 3′) and (5′ ACGGATCCCATTTTTTTATTGA 3′) incorporating the unique restriction sites *Nco*I and *Bam*HI, respectively (sites are underlined), and inserted in-frame with the downstream-located 6×His-tag-encoding sequence of the expression vector pQE60 (Qiagen), resulting in plasmid pQEamd1. Relevant sections of this plasmid were sequenced to ensure that no PCR-induced errors had occurred.

### Overexpression and purification of Amd1

An overnight culture of *E. coli* M15 carrying pQEamd1 was inoculated into 300 ml of LB medium and grown at 37 °C with vigorous shaking until an $OD_{600}$ of 0.8 was reached. The culture was cooled immediately in a static 18 °C water bath for approximately 15–20 min. The cells were then induced with 400 µM isopropyl-β-D-thiogalactoside (IPTG) and the culture grown overnight at the

**Table 1** Bacterial strains and plasmids

| Strain or plasmid | Relevant properties | Source or reference |
|---|---|---|
| Strains | | |
| *Lactococcus lactis* | | |
| MG1363 | Plasmid-free and prophage-cured Derivative of NCDO712 | Gasson (1983) |
| MG1363(pNZ8048) | $Cm^r$, $Em^r$, MG1363 harbouring plasmids pNZ8048 and pNZ9530 | This work |
| MG1363(pNZamd1) | $Cm^r$, $Em^r$, MG1363 harbouring plasmids pNZamd1 and pNZ9530 | This work |
| $[CNV]^-$ | MG1363 (Δ*pepCNV*) Unpublished | M. Hellendoorn |
| $[CNV]^-$(pNZ8048) | $Cm^r$, $Em^r$, MG1363 derivative (Δ*pepCNV*) Harbouring plasmids pNZ8048 and pNZ9530 | This work |
| $[CNV]^-$(pNZamd1) | $Cm^r$, $Em^r$, MG1363 derivative (Δ*pepCNV*) Harbouring plasmids pNZamd1 and pNZ9530 | This work |
| IM17 | MG1363 (Δ*opp*,Δ*pepO*) | I. Mierau unpublished |
| *Escherichia coli* | | |
| M15 | *E. coli* K12 derivative carrying pRep4 ($Km^r$) (*lacI*$^+$) | Qiagen |
| Plasmids | | |
| pQE60 | $Ap^r$, expression vector allowing the addition of a 6×His tag at the C terminus | Qiagen |
| pQEamd1 | $Ap^r$, 1194 bp *Nco*I–*Bam*HI Lactococcal chromosomal fragment[a] | This work |
| pNZ8048 | $Cm^r$, nisin-inducible promoter | de Ruyter et al. (1996) |
| pNZamd1 | $Cm^r$, 1194 bp *Nco*I–*Xba*I Lactococcal chromosomal fragment[b] | Curley and van Sinderen (2000) |
| pNZ9530 | $Em^r$, carries *nisR* and *nisK* | Kleerebezem et al. (1997) |

[a]Cloned between the *Nco*I and *BamH*I sites of pQE60
[b]Cloned between the *Nco*I and *Xba*I sites of pNZ8048

same temperature with vigorous shaking for approximately 16 h. This procedure prevented the formation of inclusion bodies and resulted in the production of soluble protein as judged by the presence of a 42-kDa protein in the cell-free extract of the culture when analysed on an SDS-10% polyacrylamide gel.

Purification of Amd1 was carried out at 4 °C. Cells were collected by centrifugation at 4000×*g* for 20 min and resuspended in 10 ml of ice-cold lysis buffer (50 mM sodium phosphate buffer (pH 8.0), 300 mM NaCl and 10 mM imidazole) containing lysozyme at a concentration of 1 mg $ml^{-1}$. The sample was then lysed by sonication bursts of 15 s each with 30-s intervals on ice until the solution became transparent. The resultant lysate was centrifuged at 10,000×*g* at 4 °C for 30 min and the supernatant was collected and placed on ice. A nickle/nitrilotriacetic acid column (Qiagen GmbH, Hilden, Germany) was equilibrated with 10 ml of lysis buffer, and the Amd1-containing supernatant was applied to this column. After two washing steps using 10 ml of lysis buffer containing 20 mM imidazole, protein was eluted in 10 ml of lysis buffer containing 250 mM imidazole. The eluted protein was dialysed against 2 l Tris-HCl buffer, pH 7.0 containing 1 mM DTT for 24 h with two buffer changes. Samples were analysed on a 10% SDS-PAGE gel and Amd1 was shown to be isolated as an essentially (>95% pure) homogeneous protein. The concentration of Amd1 was determined by the Bradford assay (Bradford 1976) using bovine serum albumin (Sigma) as a standard. Amd1 was stored in 40% glycerol (v/v) at –20 °C until needed.

### Enzyme activity determinations

Aminoacylase activity was measured essentially as described previously by Sakanyan et al. (1993) and Lamothe and McCormick (1973) with the following modifications. Assay mixtures of 1 ml containing the enzyme and substrate in 50 mM potassium phosphate buffer (pH 7.0) were incubated at 30 °C for 5 min. Reactions were stopped by the addition of 500 µl 10% trichloroacetic acid and precipitated protein was immediately removed by centrifugation. The supernatant solutions (1 ml) were mixed with 1 ml of hydrindantoin and heated in a boiling water bath for 15 min, followed immediately by the addition of 3 ml of ice-cold isopropanol. The absorbance at 570 nm ($A_{570}$) was measured and values were converted to µmol amino acid by reference to standard curves. One unit of aminoacylase activity is defined as the amount of enzyme that liberates 1 µmol of L-amino acid per min under these assay conditions.

Peptidase activity was determined using the Cd-ninhydrin assay (Doi et al. 1981). Assay mixtures (500 µl) containing the enzyme and substrate in 50 mM $KP_i$ buffer (pH 7.0) were incubated at 30 °C for 5 min. The reactions were terminated by the addition of 1 ml Cd-ninhydrin reagent. The contents were thoroughly mixed and heated at 84 °C for 5 min, cooled immediately on ice,and the $A_{505}$ was measured. One unit of peptidase activity is defined as the amount of enzyme that liberates 1 µmol of L-amino acid per min under these conditions.

### Determination of metal ion content of Amd1

The metal content of the purified holoenzyme was determined using inductively coupled plasma atomic emission spectrometry (ICP-AES) by A. G. Cox in the Department of Chemistry, University of Sheffield, England.

### Growth of Amd1-expressing *L. lactis* strains in CDM without leucine

Overnight cultures (in GM17) of *L. lactis* wild-type and triple peptidase mutant (carrying deletions in the aminopeptidase genes *pepC* and *pepN*, and the dipeptidase gene *pepV*) cells harboured the plasmids pNZ9530 (Kleerebezem et al. 1997) (containing *nisR* and *nisK*) and pNZamd1 (Curley and van Sinderen 2000) (with *amd1* cloned downstream of the *nisA* promoter*)*. These two plasmids in combination allowed the overexpression of Amd1 using the antimicrobial peptide nisin (de Ruyter et al. 1996). The cultures were washed twice in 0.9% NaCl before being inoculated to 2% in CDM containing all the amino acids essential for rapid growth. Subsequently, cultures were washed and inoculated into CDM which lacked the essential amino acid leucine but contained the latter in the form of 1 mM of either the dipeptide, Leu-Met, an N-acetylated amino acid (leucine) or an N-acetylated peptide containing leucine in either the first or second last position. Nisin was added at a final concentration of 10 ng $ml^{-1}$ and growth was measured by determining $OD_{600}$ values at various time points.

## Results

### Purification of *L. lactis* Amd1

*amd1* (1,194 bp) was amplified by PCR and cloned in-frame with a downstream-located 6×His-tag-encoding sequence in the *E. coli* expression vector pQE60. The protein was overexpressed in *E. coli* strain M15 cells. As determined by SDS-PAGE, nickel/nitrilotriacetic acid column affinity purification resulted in over 95% pure protein at a concentration of 0.5 mg $ml^{-1}$. The protein ran on an SDS-10% polyacrylamide gel at a molecular mass of approximately 44 kDa, which is consistent with the molecular mass deduced from the nucleotide sequence of the His-tagged *amd1* gene (44,147.64 Daltons).

### Physical properties of Amd1 and the effect of divalent metal ions on catalytic activity

The active site of aminoacylases is known to be associated with metal ions, which are essential for activity (Wu and Tsou 1993). For Amd1, metal analyses indicated that the purified aminoacylase contained only zinc and cobalt in significant amounts, approximately 2.0 g-atoms of each per subunit of enzyme. To determine the metal dependency of Amd1, divalent cations were removed by dialysis against the metal-chelating reagent EDTA. After dialysis, the protein had completely lost its activity towards its substrate N-acetyl-L-alanine; however, activity could be fully restored upon dialysis in 50 mM potassium phosphate buffer (pH 7.0) containing either 1 mM $ZnCl_2$ or

**Table 2** The effect of metal cofactors on the catalytic activity of Amd1 using the standard assay conditions described in Materials and methods. Each assay mixture contained 20 µg of Amd1, 5 mM N-acetyl-L-alanine and 1 mM of either $Co^{2+}$, $Zn^{2+}$, $Mn^{2+}$, $Mg^{2+}$ or $Cd^{2+}$. All assays were done twice and in triplicate each time. All values are average±SE

| $M^{2+}$ | $K_m$ (mM) | $V_{max}$ (µmol $min^{-1}$ $mg^{-1}$) |
|---|---|---|
| None added | 1.2±0.2 | 448±17.2 |
| $Co^{2+}$ | 0.6±0.1 | 2235±50.1 |
| $Zn^{2+}$ | 1.2±0.1 | 595±10.4 |
| $Ni^{2+}$ | 1.3±0.2 | 540±18.8 |
| $Mn^{2+}$ | 1.0±0.1 | 866±12.0 |
| $Mg^{2+}$ | 1.5±0.2 | 522±26.5 |
| $Cd^{2+}$ | 2.7±0.3 | 219± 8.0 |

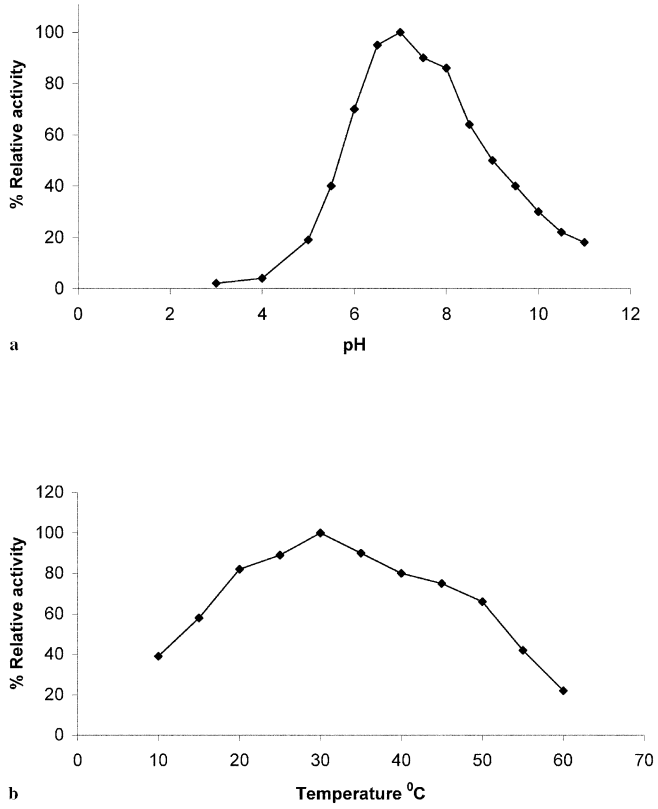


**Fig. 1** Effects of pH (**a**) and temperature (**b**) on the activity of *Lactococcus lactis* aminoacylase. **a** The effect of pH was determined in universal buffer [boric acid (57 mM), citric acid (33 mM), $NaH_2PO_4$ (33 mM), NaOH (1 M)] (Magboul and McSweeney 1999). The assay mixture contained Amd1 (20 µg), N-acetyl-L-alanine (5 mM) and 1 mM $CoCl_2$. A 100% activity level corresponds to 1,942 U mg$^{-1}$. **b** The effect of temperature was determined in the range 10–60 °C. The assay mixture contained Amd1 (20 µg), N-acetyl-L-alanine (5 mM) and 1 mM $CoCl_2$ in 50 mM $KP_I$ buffer (pH 7.0). A 100% activity level corresponds to 1,942 U mg$^{-1}$

$CoCl_2$. Other divalent cations ($Mn^{2+}$, $Mg^{2+}$, $Ni^{2+}$, $Cd^{2+}$) were ineffective. This demonstrated that $Zn^{2+}$ or $Co^{2+}$ is essential for activity.

The standard assay mixture for purified Amd1 did not contain any added metal ions, but activity could be increased significantly by the addition of $Co^{2+}$ (five-fold specific activity increase) and to a lesser extent by $Mn^{2+}$. Both metal ions enhanced catalytic activity by lowering $K_m$ values for the substrate N-acetyl-L-alanine. $Cd^{2+}$ was the only metal cofactor that was found to be inhibitory, raising the $K_m$ value for the same substrate and decreasing activity by greater than 50%. Other divalent cations ($Zn^{2+}$, $Mg^{2+}$, $Ni^{2+}$) had no significant effect (Table 2). To determine the pH and temperature optima for the enzyme, the activity of the enzyme was determined in the pH range of 3–11 (Fig. 1A), and the temperature range of 10–60 °C (Fig. 1B). The activity profiles showed that the protein, as expected, was most active at conditions found within a growing *L. lactis* cell (pH 7.0 and 30 °C).

## Substrate specificity

Amd1-catalysed deacylation appears to be L-stereospecific since the enzyme was unable to hydrolyse N-acyl-D-alanine. The α-acetyl group is exclusively deacetylated by the enzyme as judged by a lack of reactivity with ε-acetyl-L-lysine, while no activity was detected when N-acetylated peptides were used as substrates. The reaction parameters for N-acetylated amino acids depend very much on the nature of the side chain present. The enzyme hydrolyses, most efficiently, neutral aliphatic and aromatic amino acids (in particular alanine and phenylalanine). It shows poor reactivity towards polar amino acids like aspartate and glutamate, and does not appear to hydrolyse the acetylated forms of histidine and arginine (Table 3). The activity of Amd1 also varies with respect to the nature of the radical that blocks the N-terminus of the amino acid. The enzyme is able to hydrolyse three L-phenylalanine derivatives with decreasing efficiencies in the order of chloroacetyl, acetyl and benzoyl (Table 3).

Furthermore, Amd1 was shown to be catalytically active against a number of dipeptides (Table 4). $K_m$ values for these substrates were one- to 12-fold higher, while $V_{max}$ values were 14–99% of those obtained for the hydrolysis of N-acyl-amino acids. Interestingly, dipeptides with glutamate, glycine or histidine as the N-terminal amino acid were not hydrolysed. Amd1 was unable to degrade any of the tripeptides or tetrapeptides tested (results not shown).

**Table 3** Substrate specificity of Purified *L. lactis* Aminoacylase Amd1. The enzymatic activity was measured in terms of amino acid formed, by the hydrindantin-ninhydrin reaction described in Materials and methods. The steady-state kinetic parameters for 20 µg of Amd1 using a number of N-acetylated substrates were determined over the range of concentrations 1–40 mM. $K_m$ and $V_{max}$ values were calculated from Lineweaver-Burk plots. All assays were done twice and in triplicate each time. All values are average±SE

| Substrate[a] | $K_m$ (mM) | $V_{max}$ (µmol min$^{-1}$ mg$^{-1}$) |
|---|---|---|
| Nα-acetyl-L-alanine | 1.2±0.2 | 448.4±17.2 |
| Nα-acetyl-L-valine | 2.5±0.4 | 251.4±19.0 |
| Nα-acetyl-L-leucine | 2.3±0.1 | 226.2±15.7 |
| Nα-acetyl-L-isoleucine | 3.1±0.2 | 205.8±14.6 |
| Nα-acetyl-L-proline | 5.0±0.4 | 135.9±12.0 |
| Nα-acetyl-L-phenylalanine | 1.7±0.1 | 357.3±19.4 |
| Nα-acetyl-L-tyrosine | 1.9±0.2 | 339.7±16.2 |
| Nα-acetyl-L-tryptophan | 2.4±0.3 | 323.4±14.3 |
| Nα-benzoyl-L-phenylalanine | 2.7±0.3 | 309.1±25.6 |
| Nα-chloroacetyl-L-phenylalanine | 1.4±0.1 | 399.5±28.0 |
| Nα-acetyl-L-cysteine | 9.1±1.0 | 102.6±12.1 |
| Nα-acetyl-L-methionine | 7.7±0.5 | 94.4± 8.4 |
| Nα-acetyl-L-serine | 4.2±0.3 | 156.2±20.9 |
| Nα-acetyl-L-threonine | 4.0±0.8 | 183.4±14.8 |
| Nα-acetyl-L-lysine | 6.7±0.6 | 114.8± 7.0 |
| Nα-acetyl-L-glutamate | 5.9±0.4 | 138.6±10.1 |
| Nα-acetyl-L-aspartate | 5.0±0.3 | 144.7±13.2 |

[a]No detectable activity was observed with the following compounds: Nα-acetyl-L-Arg, Nα-acetyl-L-His, Nα-acetyl-D-Ala, Nε-acetyl-L-Lys, Nα-acetyl-L-Ala-Ala-Ala, Nα-acetyl-L-Met-Leu-Phe

**Table 4** Peptidase activity of Purified *L. lactis* Aminoacylase Amd1. The enzymatic activity of 20 µg of Amd1 was determined using a cadmium-ninhydrin reagent that only detects the exposed $\alpha$-$NH_2$ of amino acids and not that of peptides and proteins. Peptide substrate concentrations were varied between 1 and 40 mM, and $K_m$ and $V_{max}$ values were calculated from linear double-reciprocal plots. All assays were done twice and in triplicate each time. All values are average±SE

| Substrate[a] | $K_m$ (mM) | $V_{max}$ (µmol min$^{-1}$ mg$^{-1}$) |
|---|---|---|
| L-Alanyl-L-valine | 10.0±1.0 | 86.0±10.9 |
| L-Alanyl-L-alanine | 8.3±0.6 | 90.6± 8.8 |
| L-Leucyl-L-leucine | 9.1±0.8 | 93.0±15.2 |
| L-Leucyl-L-methionine | 11.1±2.0 | 83.9±12.3 |
| L-Leucyl-L-methionine | 14.3±2.9 | 64.7± 5.2 |
| L-Alanyl-L-tyrosine | 11.8±0.5 | 74.7± 8.5 |
| L-Phenylalanyl-L-phenylalanine | 11.2±1.0 | 73.0± 9.6 |

[a]No detectable activity was observed with the following compounds. Glu-Leu, Gly-Leu, His-Leu, Met-Leu-Phe, Ala-Ala-Ala, Leu-Gly-Gly, Lys-Trp-Lys, Phe-Gly-Phe-Gly and Gly-Pro-Phe-Gly and Gly-Pro-Gly-Gly

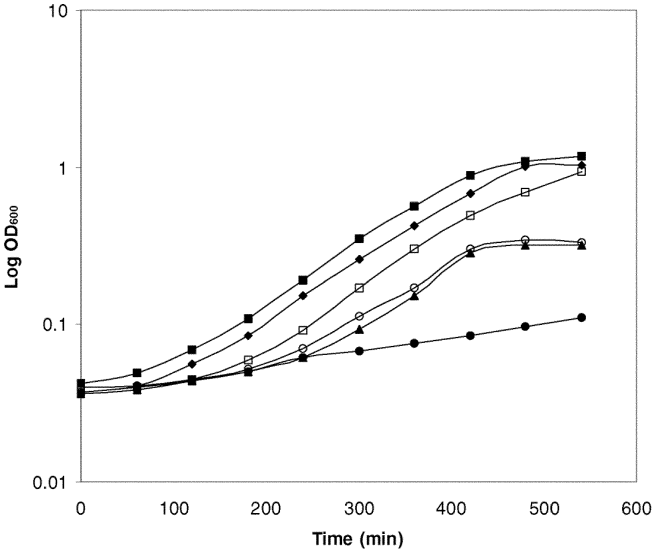


**Fig. 2** The effect of plasmid-expressed *amd1* on the growth of peptidase mutant strains of *L. lactis* in CDM lacking the essential amino acid leucine ♦ strain MG1363(pNZ8048) (+), ■ strain MG1363(pNZamd1) (+), ▲ strain [CNV]$^-$(pNZ8048) (+), □ strain [CNV]$^-$(pNZamd1) (+), ○ strain [CNV]$^-$(pNZamd1) (–) were grown in CDM without free leucine but with Leu-Met at 1 mM, and ● strain MG1363(pNZ8048) (+) was grown in CDM without free leucine and Leu-Met. + Addition of 10 ng nisin ml$^{-1}$ – absence of nisin

## Complementation of a growth deficiency in a *L. lactis* triple peptidase mutant

All dairy lactococcal strains are auxotrophic for the amino acids arginine, methionine, leucine, isoleucine, valine, glutamine and histidine (Juillard et al. 1998). Hence, in order to grow, *L. lactis* requires their presence in the surrounding environment (Fig. 2 shows that when Leu was omitted from CDM, strain MG1363(pNZ8048) was unable to grow). The triple peptidase mutant [CNV]$^-$ is severely impaired in its ability to break down dipeptides, as PepC and PepN (both of which are general aminopeptidases), as well as PepV (dipeptidase) are all involved in the breakdown of internalised (di)peptides. Therefore, growth of mutant [CNV]$^-$ in CDM, in which the only available leucine is in the form of the dipeptide Leu-Met, would be very slow. As our enzymatic assays showed, Amd1 hydrolyses Leu-Met. It was hence decided to study whether the expression of this protein in mutant [CNV]$^-$ could complement such a growth deficiency. This experiment could be carried out in a *L. lactis* MG1363 background, as the gene in the chromosome encoding Amd1 is not expressed in this strain (Curley and van Sinderen 2000). Strain [CNV]$^-$(pNZamd1) grew twice as fast and reached a three-fold higher final optical density than the control strain [CNV]$^-$(pNZ8048) which does not express *amd1* (Fig. 2). These results clearly demonstrate that Amd1 can complement the growth deficiency exhibited by such a peptidase mutant, and in turn can be seen to have a biological function in this genetic background.

Similar assays were carried out in wild-type cells using N-acetyl-L-Leu and N-acetyl-L-Leu-Arg-Arg-Ala-Ser-Val-Ala (the unmodified version of the latter peptide was shown to support the growth of strain MG1363, but not of the mutant, IM17 (Δ*opp*) ) instead of Leu-Met. However, none of the acetylated compounds was capable of supporting the growth of strain MG1363 expressing Amd1. Possibly this is due to the inability of *L. lactis* MG1363 to take up the N-acetylated forms of either amino acids or peptides. In fact, the former was already demonstrated by Driessen et al. (1987). The finding that N-acetyl-L-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln is unable to support the growth of strain MG1363 expressing Amd1 would strongly suggest that N-acetylated peptides cannot be taken up either.

## Discussion

The gene encoding the *L. lactis* MG1363 aminoacylase, *amd1*, does not appear to be expressed as judged by the lack of aminoacylase activity from lactococcal MG1363 crude extracts. The insertion of a transposon immediately upstream of the *amd1* gene is probably the reason this native enzyme is not produced (Curley and van Sinderen 2000). The presence of a paralogous gene on the same chromosome (D. van Sinderen, unpublished observation) suggests that this gene specifies a peptidase, as no aminoacylase activity can be detected from this strain. It is also noteworthy that there are two homologues of *amd1* on the genome of *L. lactis* IL1403 (Bolotin et al. 2001). The organisation of one of these homologues has been previously discussed (Curley and van Sinderen 2000).

Recombinant Amd1 was produced at levels that allowed the purification of approximately 5 mg of homogeneous protein from 300 ml of an overproducing *E. coli* culture. The enzyme displayed a subunit molecular mass of 42 kDa and is assumed to be a dimer like most aminoacylases characterised to date (the aminoacylases characterised from *Bacillus thermoglucosidius* (Cho et al. 1987) and *Py-*

*rococcus furiosus* (Story et al. 2001) are homotetramers). Amd1 was shown to behave as a metalloenzyme as evidenced by its sensitivity to the metal chelator EDTA. The assignment to this class of enzymes was also confirmed by the restorative effect exerted by the metals zinc and cobalt. The requirement for such a metal, most probably positioned at the active site, is very typical of most aminoacylases isolated and characterised to date. The enzyme, as isolated, contains zinc and cobalt in a ratio of 1:1, and is active in the absence of exogenously added metal salts. The activity of the enzyme, however, was greatly enhanced by the addition of Co(II), showing this metal to be a clear activator of the enzyme, while Zn(II) is slightly activating at the same concentration. These data suggest that each subunit of Amd1 has two metal-binding sites and is hence a member of a broad class of binuclear metallohydrolases that contain two metal ions at the active site (Wilcox 1996). For one such hydrolase, carboxypeptidase G2 ($CPG_2$) from *Pseudomonas* sp. RS-16 (Rowsell et al. 1997) which, like Amd1, is a member of the M20/M25/M40 peptidase superfamily, the 3-dimensional structure has been determined. This protein consists of a dimer harboring two zinc ions at the active site and a symmetric distribution of carboxylate and histidine ligands. An alignment of the protein sequence of Amd1 with the latter shows the conservation of an aspartate and glutamate residue (Asp-108 and Glu-141) that are most likely involved in coordination of the metal ions. The nonconserved residues, which are also predicted to be involved in coordination, include Asp-80 and His-166. Regarding substrate binding, we propose that certain hydrophobic residues within the active site, which are not conserved in $CPG_2$, are responsible for the coordination of a large number of different substrates and hence play a role in determining the broad specificity of the protein.

A preliminary study on the substrate specificity of Amd1 has previously been described (Curley and van Sinderen 2000). This report describes a detailed characterisation of the purified Amd1 enzyme using a wide variety of N-acetylated-L-amino acids. The enzyme exhibits a broad range of activity as it was shown to hydrolyse 15 of 17 N-acetylated amino acids tested. Amd1 was shown to hydrolyse both neutral aliphatic and aromatic amino acids at a very fast rate ($V_{max}$ values for alanine and tyrosine are 448.4 and 339.7, respectively). The hydrolysis of aromatic amino acids is unusual among bacterial aminoacylases and, to the best of our knowledge, has only been reported for two other enzymes, Ama from *Bacillus stearothermophilus* (Sakanyan et al. 1993) and PP from *Pyrococcus horikoshii* OT3 (Ishikawa et al. 2001). Interestingly, amino acids with anionic side chains are weak substrates ($V_{max}$ value for Aspartate is 144.7), while histidine and arginine, which possess a cationic side chain, are not hydrolysed. $K_m$ values for most substrates determined with Amd1 are comparable to those found with other aminoacylases, such as those of *E. coli* (Javid-Majd and Blanchard 2000) and *P. furiosus* (Story et al. 2001).

The fact that Amd1 was unable to hydrolyse any of the N-acetylated peptides tested for activity would seem to suggest that the enzyme exclusively removes the acyl group from amino acids and not from similarly modified peptides. In general, substrates of the latter type are only hydrolysed by an acyl-amino-acid-releasing enzyme. This enzyme catalyses the N-terminal hydrolysis of Nα-acylpeptides to release Nα-acylated amino acids (Ishikawa et al. 1998). Recently, an enzyme with both amino- and carboxy-terminal hydrolysing activities, a carboxypeptidase/aminoacylase from *P. horikoshii* OT3 (Ishikawa et al. 2001), has been discovered; however this would appear to be the first of its kind and an exception to the rule.

Amd1 also exhibited activity towards some dipeptides. The $K_m$ values for alanyl-alanine and leucyl-leucine (8.3 and 9.1 mM, respectively) reflect a similar and six-fold lower affinity, respectively, for these substrates when compared to a dipeptidase from *L. lactis* ssp. *cremoris* Wg2 (van Boven et al. 1988). For dipeptidases isolated from *Lactobacillus* strains, e.g. *Lb. delbruekii* ssp. *bulgaricus* (Wohlrab and Bockelmann 1992), the affinity for these dipeptides is higher again ($K_m$=0.56 mM for Leu-Leu). Dipeptidase activity has been previously reported for mammalian aminoacylase (Kordel and Schneider 1975; Heese et al. 1988; Moravcsik et al. 1997), albeit only potent at higher pHs (above pH 8.0), and some bacterial aminoacylases (Sakanyan et al. 1993; Ishikawa et al. 2001). This dipeptidase activity was suggested to occur as a result of the replacement of the acyl moiety of the amino acid by the N-terminal amino acid of the dipeptide as a target for hydrophobic interactions with active site regions (Kordel and Schneider 1975). Furthermore, dipeptidases share various properties with aminoacylases: their subunit molecular weight, metal content and requirement, amino acid composition, and amino acid sequence in the N-terminal region are very similar (Cho et al. 1988). This may indicate that dipeptides are the true substrates of aminoacylases. In fact, we demonstrate that the dipeptidase activity of Amd1 plays a physiological/nutritional role within the bacterial cell in a specific genetic background (i.e. that of a multiple peptidase mutant). We were unable to demonstrate whether Amd1 is biologically active towards either acetylated amino acids or acetylated peptides. This can be explained, however, by the inability of *L. lactis* to transport these modified derivatives into the cell, perhaps due to the absence of a specific transporter system for these particular compounds. Hence, it may be that acetylation of proteins and/or peptides occurs inside the cell, perhaps as a signal for further digestion (once they are internalised) or even as some sort of post-translational modification mechanism. Amd1 may therefore have (had) two biological functions. The first may be as a dipeptidase, whose loss due to transposon disruption was compensated by other peptidases. The second function may involve turnover of acetylated amino acids and/or peptides, once they have been signalled for digestion by the addition of an acetyl group. This would also make sense when assuming that the acetylated forms of such compounds are not transported across the membranes of *L. lactis* and thus must be formed within the cell.

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